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Purification and Properties of a Thermostable Chitinase from *Streptomyces thermoviolaceus* OPC-520

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A chitinase was purified from the culture filtrate of *Streptomyces thermoviolaceus* OPC-520. The enzyme showed a high optimum temperature (70 to 80°C), a high optimum pH level (8.0 to 10.0), and heat stability. This enzyme showed high sequence homology with chitinases from *Serratia marcescens* QMB1466 and *Bacillus circulans* WL-12.

Chitinase (EC 3.2.1.14) plays an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. Practical applications of chitinase include its use in the preparation of protoplasts from fungi (28), as a protective agent against plant-pathogenic fungi (18), and in the production of oligosaccharides as biologically active substances (26). Thus, chitinolytic enzymes have been purified from many microorganisms, and their enzymatic properties have been investigated. *Streptomyces* spp. make up one group regarded as particularly efficient in the breakdown of chitin (2). Previously, we purified and characterized thermostable xylanases produced by *Streptomyces thermoviolaceus* OPC-520 (23). Recently, we found that this microorganism also produced a thermostable chitinase when grown in a medium containing colloidal chitin as a carbon source. This article describes the purification and properties of chitinase from a culture filtrate of *S. thermoviolaceus* OPC-520.

The strain produced relatively high levels of chitinase activity (1.13 U/ml of culture filtrate) when grown in a medium (100 ml) (containing the following [g/liter]: proteose peptone [Difco], 5; yeast extract [Difco], 1; colloidal chitin [Nakarai Tesque, Kyoto, Japan], 5 [wet weight]; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.2 [pH 7.0]) in a 500-ml flask and cultured at 50°C on a reciprocal shaker for 24 h. Chitinase (*S. thermoviolaceus* [ST] chitinase) was purified from the culture filtrate by the successive column chromatographies of DEAE-Toyopearl 650M (50 mM acetate buffer, pH 5.0; Tosoh Co., Tokyo, Japan), Sephadex G-75 (50 mM acetate buffer containing 0.1 M NaCl, pH 5.0; Pharmacia), phenyl-Toyopearl 650M [50 mM acetate buffer containing 1 M $(NH_4)_2SO_4$; Tosoh Co.], and Mono-Q HR (50 mM acetate buffer, pH 5.0; Pharmacia). Protein was estimated by the method of Lowry et al. (10) with bovine serum albumin as the standard. The purification procedure is summarized in Table 1. In this procedure, ST chitinase was purified 20.6-fold, and the activity was 29.9% of the culture filtrate. The molecular mass of ST chitinase was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9) to be 40 kDa (Fig. 1). The isoelectric point of the enzyme (3.8) was measured by isoelectric focusing in an immobilized gradient of pH 3 to 5 (23). Recently, thermostable chitinases from *Bacillus licheniformis* X-7u were purified and characterized (20). The molecular mass and amino-terminal sequence of ST chitinase are clearly different from those of chitinases I (89 kDa, E-C-P-D-N-P), II (76 kDa, D-S-G-K-N), III (66 kDa, D-S-G-K-N), and IV (59 kDa, D-S-G-K-N) from *B. licheniformis*. Purified ST chitinase also exhibited higher specific activity (82.5 U/mg of protein) than chitinase I (4.2 U/mg of protein), which shows the highest specific activity among the enzymes from *B. licheniformis*.

Chitinase was assayed by mixing a 0.5-ml aliquot of appropriately diluted enzyme with 1.0 ml of 0.2% (wt/vol) glycol chitin (Seikagaku Corporation, Tokyo, Japan) in 50 mM Tris-HCl buffer, pH 9.0. After incubation at 70°C for 10 min, the reaction was terminated by cooling the reaction mixture in an ice bath. The reducing end groups produced were measured colorimetrically with ferric ferrocyanide reagent by a modification of the Schales procedure (4). One unit of chitinase was defined as the amount of enzyme that released 1 μmol of GlcNAc per min under these conditions.

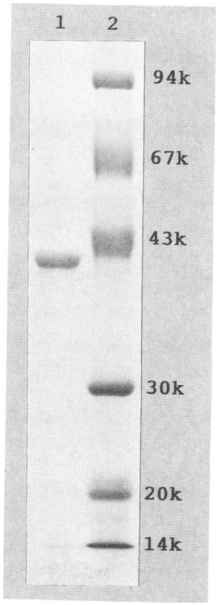

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ST chitinase. Lane 1, ST chitinase. Lane 2, molecular mass markers (in kilodaltons).

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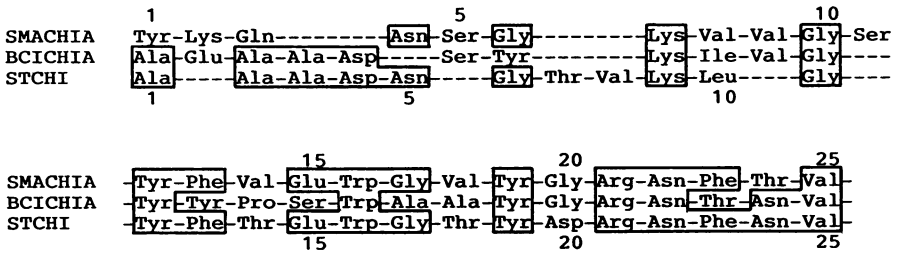

FIG. 2. Comparison of the amino-terminal sequence of ST chitinase with those of other microbial chitinases. SMACHIA, BCICHIA, and STCHI are chitinases from *Serratia marcescens* QMB1466, *Bacillus circulans* WL-12, and *Streptomyces thermoviolaceus* OPC-520, respectively. Dashed lines represent spaces inserted into sequences for alignment. The numbers below the sequences refer to ST chitinase. Amino acids identical among ST chitinase and other chitinases are boxed.

McIlvaine buffer (pH 4 to 7) (11), 50 mM Tris-HCl buffer (pH 8 to 9), and 50 mM glycine-NaOH buffer (pH 10 to 12) were used for studying the effects of pH and temperature on chitinase activity and stability. The optimum pH level was pH 9.0 and the optimum temperature was 80°C when chitinase activity was examined with 50 mM Tris-HCl buffer (pH 9.0). These values were very similar to those of thermostable chitinases (optimum pH, 10; optimum temperature, 80°C) from *B. licheniformis* X-7u (20). These properties are unique in comparison with those of other *Streptomyces* chitinases (1, 3, 13, 22, 24). For studying the effects of pH and temperature on chitinase stability, the enzyme solution was incubated for 30 min under various conditions. ST chitinase showed the remaining activity to be more than 80% in the range of pH 4 to 12 at up to 60°C when activity of the intact enzyme was taken as 100%. Furthermore, ST chitinase retained its original activity during incubation in 50 mM Tris-HCl, pH 7.0, for 14 days at 50°C. The effects of metal ions and chemical reagents on the enzymes were examined. Cu^{2+}, Hg^{2+}, Ba^{2+}, Cd^{2+}, and Sn^{2+} inhibited enzyme activity at a concentration of 1 mM. Other metal ions had no marked effect on the enzyme. ST chitinase was almost completely inhibited by *N*-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide (modification of tryptophan), and Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulfonate, modification of the carboxy group) as a protein modifier. These facts indicate that a tryptophan residue(s) and a carboxy group(s) of asparatic acid and/or glutamic acid are probably essential for the activity of ST chitinase.

The enzyme hydrolyzed chitooligosaccharides (Seikagaku Corporation) from a trimer to a hexamer to give *N*,*N*′-diacetyl-chitobiose [$(GlcNAc)_2$] as the main product but did not further hydrolyze $(GlcNAc)_2$. The hydrolysis products were analyzed by high-performance liquid chromatography in accordance with the method of Usui et al. (25). These results indicate that ST chitinase hydrolyzes chitin by an endo-activity mechanism. The enzyme did not show lysozyme activity with *Micrococcus lysodeikticus* as a substrate (5).

TABLE 1. Purification of ST chitinase

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)
Culture filtrate	221.0	884	4.0	100.0
DEAE-Toyopearl 650M	21.6	505	23.4	57.1
Sephadex G-75	4.0	311	77.8	35.2
Phenyl-Toyopearl 650M	3.6	288	80.0	32.6
Mono-Q HR 5/5	3.2	264	82.5	29.9

Usui et al. reported that chitinases purified from culture filtrates of *Nocardia orientalis* IFO 12806 (25) and *Trichoderma reesei* KDR-11 (26) possessed transglycosylation activity. ST chitinase was examined for this activity in accordance with the method of Takayanagi et al. (20). The enzyme converted 326 μg of $(GlcNAc)_4$ into 32 μg of $(GlcNAc)_6$ and 58.1 μg of $(GlcNAc)_2$ as the predominant products in a reaction time of 20 min. As time proceeded, the amount of $(GlcNAc)_6$ decreased and those of $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_5$ increased. $(GlcNAc)_6$ was reported to show immunoadjuvant effects such as activation of phagocytes (19) and a growth-inhibitory effect on solid tumors (21). Thus, the enzymatic approach to the production of $(GlcNAc)_6$ is very attractive as the alternative method of partial acid hydrolysis of chitin (16).

The amino-terminal amino acid sequence of ST chitinase (A-A-A-D-N-G-T-V-K-L-G-Y-F-T-E-W-G-T-Y-D-R-N-F-N-V) was determined on an Applied Biosystems model 477A gas phase sequencer. In this region there was sequence homology with *Serratia marcescens* chitinase (68% homology) (6) and *Bacillus circulans* chitinase (64% homology) (27), as shown in Fig. 2. However, ST chitinase was clearly different from other *Streptomyces* chitinases (7, 14, 15), *Saccharomyces* chitinase (8), and plant chitinases (12, 17), although the sequences were much too short for a completely definitive conclusion. We have succeeded in the gene cloning and expression of ST chitinase in *Escherichia coli* and are analyzing the gene structure. Further research is planned to deduce the complete primary structure of the protein from the nucleotide sequence of the gene and to investigate the relationship between the structure and function of the enzyme.

REFERENCES

1. **Berger, L. R., and D. M. Reynolds.** 1958. The chitinase system of a strain of *Streptomyces griseus*. Biochim. Biophys. Acta **29**:522–534.
2. **Gooday, G. W.** 1990. The ecology of chitin decomposition. Adv. Microb. Ecol. **11**:387–430.
3. **Hara, S., Y. Yamamura, Y. Fujii, T. Mega, and T. Ikenaka.** 1989. Purification and characterization of chitinase produced by *Streptomyces erythraeus*. J. Biochem. **105**:484–489.
4. **Imoto, T., and K. Yagishita.** 1971. A simple activity measurement of lysozyme. Agric. Biol. Chem. **35**:1154–1156.
5. **Jolles, P.** 1962. Lysozymes from rabbit spleen and dog spleen. Methods Enzymol. **5**:137–140.
6. **Jones, J. D. G., K. L. Grady, T. V. Suslow, and J. R. Bedbrook.** 1986. Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. EMBO J. **5**:467–473.

7. **Kamei, K., Y. Yamamura, S. Hara, and T. Ikenaka.** 1989. Amino acid sequence of chitinase from *Streptomyces erythraeus*. J. Biochem. **105:**979–985.
8. **Kuranda, M. J., and P. W. Robbins.** 1991. Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. J. Biol. Chem. **266:**19758–19767.
9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
11. **McIlvaine, T. C.** 1921. A buffer solution for colorimetric comparison. J. Biol. Chem. **49:**183–183.
12. **Metraux, J. P., W. Burkhart, M. Moyer, S. Dincher, W. Middlesteadt, S. Williams, G. Payne, M. Carnes, and J. Ryals.** 1989. Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc. Natl. Acad. Sci. USA **86:**896–900.
13. **Miyashita, K., T. Fujii, and Y. Sawada.** 1991. Molecular cloning and characterization of chitinase genes from *Streptomyces lividans* 66. J. Gen. Microbiol. **137:**2065–2072.
14. **Robbins, P. W., C. Albright, and B. Benfield.** 1988. Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli*. J. Biol. Chem. **263:**443–447.
15. **Robbins, P. W., K. Overbye, C. Albright, and J. Pero.** 1992. Cloning and high-level expression of chitinase-encoding gene of *Streptomyces plicatus*. Gene **111:**69–76.
16. **Rupley, J. A.** 1964. The hydrolysis of chitin by concentrated hydrochloric acid, and the preparation of low-molecular-weight substrates for lysozyme. Biochim. Biophys. Acta **83:**245–255.
17. **Shinshi, H., D. Mohen, and F. Meins, Jr.** 1987. Regulation of a plant pathogenesis-related enzyme. Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc. Natl. Acad. Sci. USA **84:**89–93.
18. **Sundheim, L., A. R. Polawsky, and A. H. Ellingboe.** 1988. Molecular cloning of two chitinase genes from *Serratia marcescens* and their expression in *Pseudomonas* species. Physiol. Mol. Plant Pathol. **33:**481–491.
19. **Suzuki, K., A. Tokoro, Y. Okawa, S. Suzuki, and M. Suzuki.** 1986. Effect of N-acetylchito-oligosaccharides on activation of phagocytes. Microbiol. Immunol. **30:**777–787.
20. **Takayanagi, T., K. Ajisaka, Y. Takiguchi, and K. Shimahara.** 1991. Isolation and characterization of thermostable chitinases from *Bacillus licheniformis* X-7u. Biochim. Biophys. Acta **1078:** 404–410.
21. **Tokoro, A., N. Tatewaki, K. Suzuki, T. Mikami, S. Suzuki, and M. Suzuki.** 1988. Growth-inhibitory effects of hexa-N-acetylchitohexaose and chitohexaose against Meth-A solid tumor. Chem. Pharm. Bull. **36:**784–790.
22. **Tominaga, Y., and Y. Tsujisaka.** 1976. Purification and some properties of two chitinases from *Streptomyces orientalis* which lyse *Rhizopus* cell wall. Agric. Biol. Chem. **40:**2325–2333.
23. **Tsujibo, H., K. Miyamoto, T. Kuda, K. Minami, T. Sakamoto, T. Hasegawa, and Y. Inamori.** 1992. Purification, properties, and partial amino acid sequences of thermostable xylanases from *Streptomyces thermoviolaceus* OPC-520. Appl. Environ. Microbiol. **58:**371–375.
24. **Ueno, H., K. Miyashita, Y. Sawada, and Y. Oba.** 1990. Purification and some properties of extracellular chitinase from *Streptomyces* sp. S-84. J. Gen. Appl. Microbiol. **36:**377–392.
25. **Usui, T., Y. Hayashi, F. Nanjo, K. Sakai, and Y. Ishido.** 1987. Transglycosylation reaction of a chitinase purified from *Nocardia orientalis*. Biochim. Biophys. Acta **923:**302–309.
26. **Usui, T., H. Matsui, and K. Isobe.** 1990. Enzymatic synthesis of useful chito-oligosaccharides utilizing transglycosylation by chitinolytic enzymes in a buffer containing ammonium sulfate. Carbohydr. Res. **203:**65–77.
27. **Watanabe, T., K. Suzuki, W. Oyanagi, K. Ohnishi, and H. Tanaka.** 1990. Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type III homology units of fibronectin. J. Biol. Chem. **265:**15659–15665.
28. **Yabuki, M., Y. Kasai, A. Ando, and T. Fujii.** 1984. Rapid method of converting fungal cells into protoplast with a high regeneration frequency. Exp. Mycol. **8:**386–390.